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Assessing the Presence and Concentration of Microplastics in the Gizzards of Virginia Waterfowl

Thomas Bustamante

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by

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Thesis

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Signature Page

This Thesis by Thomas Bustamante is accepted in its present form as satisfying the thesis requirement for Honors in Biology.

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Biography

Thomas Bustamante

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Wildlife Society Virginia Chapter Meeting • Virtual • February 2021

CPRC SETAC Annual Meeting • Virtual • September 2020

Summer Science Symposium • Fredericksburg, Virginia • July 2020, July 2018

CPRC HDC Joint Spring 2020 Meeting • Newark, Delaware • April 2020 (Conference Cancelled)

UMW Research and Creativity Day • Virtual • April 2020

Association of Southeastern Biologists Annual Meeting • Jacksonville, Florida • March 2020 (Conference Cancelled)

First Annual Fall Research Symposium • Fredericksburg, Virginia • December 2019

SETAC Annual Meeting • Toronto, Canada • November 2019

Research Experience for Undergraduates Symposium • Alexandria, Virginia • October 2019

Disturbance Ecology REU Symposium • Richmond, Kentucky • July 2019

Meeting of the Virginia, West Virginia and Virginia Tech Chapters of the American Fisheries Society • Blacksburg, Virginia • February 2019

Awards and Honors

President's List

Dean's List

Chi Beta Phi: Kappa Sigma Chapter

Phi Beta Kappa Honor Society

Rebecca Culbertson Stuart Memorial Scholarship

Sally Brannan Hurt '92 Study Abroad Biology Scholarship

Presidential Award

Taking Flight Scholarship

Summer Science 1st Best Poster

Summer Science 2nd Best Presentation

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Abstract

Microplastics are defined as plastic fragments smaller than 5mm which originate from sources such as manufactured pellets, personal care products, and the breakdown of larger plastic items. They have become a ubiquitous water pollutant in recent years, and while a substantial amount of research on their impacts on marine ecosystems has been conducted, the presence of microplastics in freshwater systems and organisms remains less understood. In this study, we assessed the presence and concentrations of microplastic particles in the gizzards of the Canada Goose (*Branta canadensis*), Longtailed Duck (*Clangula hyemalis*), Ringneck Duck (*Aythya collaris*), Mallard (*Anas platyrhynchos*), and Goldeneye Duck (*Bucephala clangula*) hunted in the Piedmont and Coastal Plain of Virginia. Gizzards were bisected, then their contents were removed for analysis. Internal gizzard contents were digested in 30% hydrogen peroxide with an iron catalyst, then were density separated in a NaCl saline solution to isolate microplastics. Samples were then visually inspected under a dissecting microscope. After laboratory contamination was taken into account, 53.6% of gizzards contained microplastics. Samples ranged in concentration from 0 to 1.75 plastics/gram of gizzard material. While concentrations did not differ between sex and location, diving ducks had significantly higher microplastic concentrations than Canada Geese. The raw number of microplastics between the two groups was the same. This result may be due to differences in the diet between diving ducks and Canada Geese. These results provide evidence that freshwater species of waterfowl not only consume microplastics, but also retain them in their digestive tracts. Results were fairly high compared to studies assessing birds in more remote areas, suggesting the level of urbanization in our sites led to these results. As microplastics continue to release into the environment, more organisms, such

as these waterfowl, will consume these plastics and potentially suffer toxicological consequences.

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Introduction

Microplastics have attracted increasing attention by researchers in recent years and are being called a contaminant of emerging concern (Wagner et al., 2014). These particles are defined as any piece of plastic that is <5mm in size, and they come in a diverse array of shapes, sizes, colors, and polymer types (Rochman et al., 2019). In general, there are two commonly recognized groupings of microplastics: primary and secondary microplastics. Primary microplastics are manufactured to be smaller than 5mm and include microbeads in facial cleansers as well as pre-production pellets used for plastic manufacturing. Secondary microplastics results from the breakdown of large plastic objects via photodegradation by ultraviolet radiation as well as physical degradation by abrasion (Horton et al., 2017).

Microplastics have been heavily studied in marine settings, whereas less focus has been put on freshwater environments until relatively recently (Driedger et al. 2015). In marine environments, microplastics are known to concentrate along coastlines, near nutrient upwells, and within large oceanic gyres (Cole et al. 2011). Marine microplastic concentrations heavily vary depending on geographic location. For example, concentrations of 0.024-0.209 mg/m^3 were found in the surface waters of the Southeast Bering Sea near Alaska (Doyle et al. 2011), while concentrations of 64-30,169 g/km^2 were found in the nearby North Pacific Gyre, sometimes outnumbering concentrations of plankton (Moore et al. 2001). Areas close to Virginia, such as the Chesapeake Bay, are known to contain concentrations as high as 1.245 $particles/m^3$ in its surface waters. However, the study investigating this also included non-plastic anthropogenic debris, which made up 10% of samples (Bikker et al. 2020).

While they have not been studied to the same extent as marine ecosystems, microplastics have still been documented in freshwater systems such as rivers (Lechner et al. 2014; Vermaire

et al. 2017), lakes (Anderson et al. 2017; Free et al. 2014), wetlands (Li et al. 2018), and the Great Lakes (Eriksen et al. 2013). Factors impacting where microplastics concentrate and originate from in freshwater systems remain more elusive and may include the level of urbanization in a given area, the size and water residence time of a given water body, waste management strategies, as well as physical factors such as wind currents and level of rain (Eerkes-Medrano et al. 2015). A common source of microplastics in freshwater environments is wastewater treatment plants. A study assessing plants across the United States found that one of these facilities discharge microplastics in concentrations between 0.004 and 0.195 particles per liter in their effluent (Mason et al. 2016). When multiplied by the volume of water each plant processes per day, the number of plastics released by these plants may be as high as $1.5x10^7$ particles per day. Results from actual freshwater studies corroborate these potential sources of microplastic pollution. For example, concentrations in the Ottawa River more than doubled from 0.71 fragments/m^3 upstream of a wastewater treatment plant to 1.99 fragments/m^3 downstream of the plant, thus suggesting the plant significantly contributed plastic pollution to the environment (Vermaire et al. 2017). Also, within Lake Winnipeg, concentrations of plastics in 2014 were significantly higher in the northern basin as opposed to the southern basin, but this pattern was not maintained in 2015 or 2016 (Anderson et al. 2017). The explanation the authors suggested was that the northern basin received effluent from much more densely populated areas than the southern basin. Even in remote freshwater lakes, such as Lake Hovsgol in Mongolia, microplastics are present in average concentrations of 20,264 particles/km^2, which rivals concentrations in the great lakes (Free et al. 2014). While nearby large human populations do not explain these results, these values may be partially explained by the small surface area and long water residence of the lake.

Microplastics are known to be readily consumed by a wide array of organisms such as aquatic invertebrates (Windsor et al. 2019), fish (Sanchez et al. 2014), birds (Basto et al. 2018), and large marine mammals such as baleen whales (Besseling et al. 2015). In laboratory studies, consumption of microplastics has been shown to cause a wide array of negative impacts for organisms (Ma et al. 2019; Wright et al. 2013). First, microplastics may cause intestinal damage to animals, as has been shown in a study assessing zebrafish. After exposure, microplastics physically caused histopathological alterations such as the cracking of villi and splitting of enterocytes (Lei et al. 2018). Additionally, microplastics are capable of efficiently transporting various hydrophobic organic pollutants such as polychlorinated biphenyls (PCBs), Dichlorodiphenyldichloroethylene (DDEs), and organochlorine pesticides on account of their high surface area to volume ratio (Horton et al. 2017). In addition, microplastics are capable of releasing various plasticizers, or plastic additives, into the environment (Wagner et al. 2014). This means that microplastics are not only capable of contaminating relatively pristine areas with pollutants, but also transporting these pollutants into organisms. This effect has already been demonstrated by studies assessing carcasses of seabirds such as the short-tailed shearwater, which contained toxic compounds in their adipose tissue that originated from plastics in their stomachs (Tanaka et al. 2013). Microplastics can also negatively impact growth and fecundity, as shown in studies assessing different species of invertebrates, such as a species of amphipod (*Hyalella azteca)* and a nematode (*Caenorhabditis elegans)* (Au et al. 2015, Lei et al. 2018).

Microplastics may even act as a selection pressure on organisms. A study assessing microplastic consumption by anemonefish found that anemonefish with higher activity levels consumed more plastics than those with lower activity levels, suggesting that they are more at risk of negative impacts on fitness (Nanninga et al. 2020). In addition, microplastics may pass

through the gut epithelium of organisms, concentrate in tissues, and thereby be transferred between trophic levels on account of their small size (Wright et al. 2013). This hypothesis has been corroborated by laboratory studies. In Daphnia, microplastics were shown to not only enter the gastrointestinal tract, but transfer through the gut epithelium to storage oil droplet cells after 24 hours of exposure (Rosenkranz et al. 2009). Another study assessing trophic transfer of plastics between mussels and crabs showed that following the consumption of mussels that were exposed to microplastics, crabs retained small amounts of plastics in their stomachs, hepatopancreases, ovaries, and gills despite never being directly exposed to microplastics themselves (Farrell and Nelson 2013).

Ingestion of microplastics by many marine birds, especially the Northern Fulmar (Provencher et al. 2018), has been studied extensively compared to studies of freshwater birds. The few studies that have been carried out have revealed widespread microplastic ingestion by freshwater species (Brookson et al. 2019; Gil-Delgado et al. 2017; Holland et al. 2016; Reynolds and Ryan 2018; Winkler et al. 2020). Most of these studies assessed fecal material, gizzard contents, as well as portions of or the entire gastrointestinal tract. One study examined regurgitation pellets from the Common Kingfisher (*Alcedo atthis*) (Winkler et al. 2020). Species found with microplastics in their system include various waterfowl (Reynolds and Ryan 2018), the Common Kingfisher (Winkler et al. 2020), and the Double-Crested Cormorant (*Phalacrocorax auritus*) (Brookson et al. 2019). These birds have been assessed in many locations, including South Africa, Italy, and Canada. However, microplastic ingestion by waterfowl in the Mid-Atlantic United States is unstudied. Additionally, previous studies reported their results as the raw numbers of microplastic particles as opposed to concentration of particles per unit dry sample mass, without controlling for total amount of material in the digestive tract.

This could lead to inaccurate interpretations of data. For example, if one bird eats twice as much as another, but they have the same number of microplastics in their gastrointestinal tract, then microplastics are probably less prevalent in the first bird's environment. This means there would likely be different implications for each bird that are not taken into account by only reporting particle count. With that in mind, the raw counts ranged from as few as 82 (Reynolds and Ryan 2018) to as many as 736 (Gil-Delgado et al. 2017). Most of these particles were secondary fibers and fragments. Previous studies have also revealed a high range in frequency of occurrence, or percentage of birds with particles in their system. Percentages ranged from 4.3% (Holland et al. 2016) to 86.7% (Brookson et al. 2019). Some studies grouped all anthropogenic particles found together in this parameter, which makes these percentages less reflective of microplastics ingestion, specifically. As studies move forward, they should report diverse metrics in their results in order to allow for comparison between studies as well as report concentrations of plastics as opposed to raw count so amount of food consumed can be taken into account.

In this study, we quantified the presence of microplastics in the gizzards of the Canada Goose (*Branta canadensis*), Long-tailed Duck (*Clangula hyemalis*), Ringneck Duck (*Aythya collaris*), Mallard (*Anas platyrhynchos*), and Goldeneye Duck (*Bucephala clangula*) hunted in the Piedmont and Coastal Plain of Virginia. Specifically, samples came from Westmoreland and Culpeper County, Virginia. The ducks and geese studied here vary in feeding ecology. One of our species (the Mallard) is a dabbling duck, three species (Longtailed Duck, Ringneck Duck, and Goldeneye Duck) are diving ducks, and the Canada Goose has its own feeding habits. Dabbling ducks skim their bills through the water and feed on surface plants, while diving ducks dive underwater to consume prey and plants (Raikow 1973). Canada Geese typically consume terrestrial grasses and grains, depending on the time of year, but consume aquatic grasses as well

(Mowbray et al. 2002). These differences in feeding, as well as location and sex, could lead to differences in microplastic consumption.

Overall, we predicted that microplastic concentrations would be higher for birds collected from Westmoreland County than Culpeper County because Westmoreland borders the Potomac River, which is downstream of highly populated areas like Washington DC. We also predicted that dabbling ducks would have higher concentrations than diving ducks or Canada Geese since their feeding strategy involves filtering water as opposed to targeting specific food items. Not many microplastic studies have compared sexes. However, studies looking at marine bird macroplastic consumption have found no significant differences in the past (Spear et al. 1995). Therefore, we expected to see no major differences as well, but microplastic-specific differences must still be assessed. We stress our utilization of concentration per unit food residue mass in addition to raw microplastic count. While the raw number of plastics does provide some measurement of microplastic prevalence, concentration takes the amount of food a given bird has consumed into account, therefore providing a more accurate measurement of relative ingestion.

Materials and Methods

Sample Collection

We obtained whole waterfowl carcasses from hunters in January of 2019 and 2020, who collected them in Culpeper and Westmoreland County, Virginia (Fig. 1). We also obtained additional preprocessed Canada Goose gastrointestinal tracts hunted in Westmoreland County. The Canada Goose is a partial migrant in that some populations migrate while others do not (Mowbray et al., 2002). The Canada Geese hunted in Culpeper County were permanent residents as indicated by their higher body fat and fatty liver while the geese hunted in Westmoreland were likely from the James Bay migratory population based on a USFWS bird banding recovery

(Truong W. personal communication). This method of sampling birds likely decreased bias compared to haphazard collection of carcasses found in the environment since the birds that have been killed naturally likely die from factors such as poor body condition or sickness. A study testing this idea found that Short-Tailed Shearwater fledgling carcasses collected on the beach had higher plastic loads than those killed by humans on the road (Rodríguez et al., 2018). Thus, by using birds that were active at the time they were hunted, we likely reduced bias toward unhealthy or otherwise compromised birds in our study.

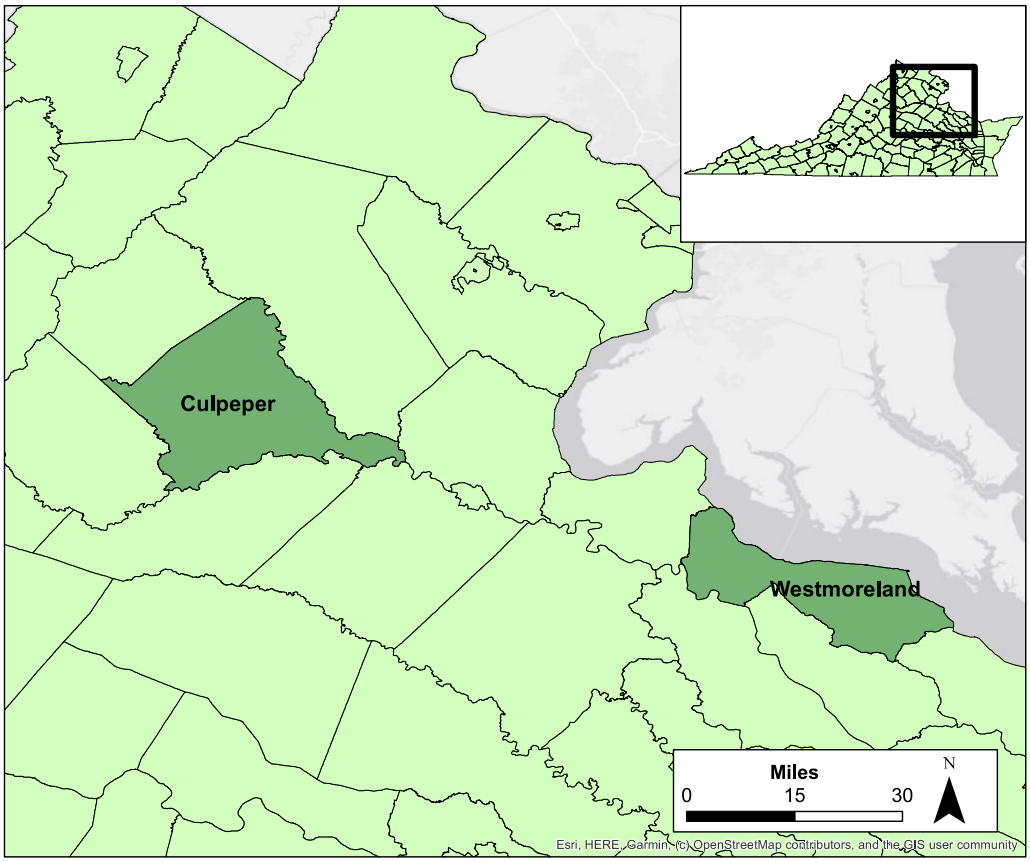

Figure 1: Map of our two sample counties: Culpeper and Westmoreland county. Birds from these counties were donated to us by hunters. Culpeper county is farther inland, at a higher elevation, and has a higher human population than Westmoreland. Westmoreland is lower in population, but is downstream of many populated areas such as Washington DC. Virginia County shapefile from ArcGIS.com.

Sample Storage

We either dissected or immediately froze bird carcasses at ~ -20°C from the hunters for later processing. Upon dissection, we removed each bird's gizzard, intestines, and liver, fat, and muscle tissue samples. We determined each individual's sex via inspection of plumage or gonads. We stored gizzard samples in bags made from compostable corn starch and froze them at ~ -20°C once again until further processing. Some Canada Goose carcasses hunted in Westmoreland County were pre-dissected by the donor, and we received only the gastrointestinal tracts (n=12). We were therefore unable to determine the sex of these individuals. These samples were sent in plastic Ziplock bags, and we immediately stored these samples in a freezer until later processing.

The gizzard is a powerful muscular organ that specializes in breaking down food that birds consume (Svihus 2011). This organ plays a crucial role in digestion, and it is relatively easy to sample from as opposed to other parts of the digestive tract. Additionally, microplastics are likely to be retained for prolonged periods in this organ. Therefore, we chose to extract samples of food residue from gizzards to index microplastic ingestion.

Laboratory Contamination Control

Sample contamination can be a problem in microplastics studies. Microplastics freely floating in the air, in chemical reagents and water systems, and on people's hands or clothes can easily contaminate samples and lead to overestimation in results (Dehault et al., 2019). Thus, we took precautions to reduce contamination of our samples. We stored samples in non-plastic containers such as glass vials and compostable bags made of cornstarch. We wore lab coats and nitrile gloves at all times during sample processing and quantification. We covered samples with aluminum foil whenever they were not being directly worked on. We processed all gizzards in a

fume hood, and wiped the fume hood's surface with nanopure water before and after use. We washed all equipment with soap and deionized or nanopure water before and after every use. We used a hot pink sponge to clean equipment, and thus hot pink plastics were excluded from analysis. Before use, we filtered all solutions through a sieve and used nanopure water instead of the deionized water system. Finally, we processed a set of procedural blanks alongside gizzard samples. We processed these blanks just as the samples above, except an empty beaker was processed alone without any gizzard material. In order to take this laboratory contamination into account, we calculated the average number of plastics in each color and shape category among all of the blanks. We then selectively subtracted those averages from each sample based on the color and types of plastics present. If one plastic remained following this subtraction that fit into a category of plastic found in the blank, then we also subtracted it. If more than one plastic remained, we assumed it to have originated from the gizzard.

Microplastic Extraction

Before processing, gizzards were removed from the freezer and allowed to thaw for 24 hours. Once thawed, we bisected each gizzard and removed three samples of its internal contents with a metal scoop. Each sample varied in mass depending on if it came from a duck (0.86±0.3g) or a goose (3.23±1.15g) given the sizes of their gizzards. Internal contents were mostly grit, but also food material which varied depending on the diet of the bird. We placed each portion in a separate beaker, covered the beaker with aluminum foil, and placed it in a drying oven at ~106°C for 12 hours. We then recorded the dry mass of each sample of gizzard contents by subtracting the mass of the sample in its beaker by the mass of the empty beaker.

Once the gizzard sample was dried and massed, it was chemically digested and density separated following methods from Masura et al. (2015). This method has been used to assess

water and beach sand samples, but because gizzards contain a large amount of grit for breaking down food, we processed these samples as sediment samples. First, we added 20mL of 30% hydrogen peroxide and an iron catalyst to each beaker containing a gizzard contents sample. We prepared this iron catalyst in the lab by mixing 500mL of nanopure water with 3mL of concentrated sulfuric acid and 7.5g of FeSO4°7H20. We allowed the sample to react for five minutes. Following the five-minute period, we added a magnetic stir bar to each beaker and transferred the samples to a hotplate that was set to 75°C. We then stirred the samples on the hotplate for 30 minutes. If an abundance of organic material remained, we added an additional 20mL of hydrogen peroxide and the process was completed a second time. Following digestion, we added about 6g of NaCl to the solution for every 20mL of liquid in the sample. This allowed us to increase the density of the solution (~5 M NaCl) for density separation. We stirred the saline solution on the hotplate at 75°C until all of the salt dissolved. We then removed the stir bar, and allowed the beaker to settle overnight. We did not use the same density separator as Masura et al. (2015), as the settled sediment in gizzards was too coarse and did not allow for easy drainage of the liquid portion without significant loss of the sample. Instead, we decanted the top layer of the beaker into a vacuum filter directly. The filter papers used were Fisherbrand P4 grade filter papers. They had a diameter of 5.5cm, were composed of cellulose fibers, and were manufactured to retain particles as small as 4-8 μm. We stored these filter papers in small plastic petri dishes so they would be covered from airborne contamination and allowed them to dry overnight at room temperature prior to microplastic quantification.

Microplastic Quantification

Once the filter paper was dry, we counted microplastics. We followed the filter paper counting method described by the Marine Environmental Research Institute (no date). We

counted each paper by sliding the paper from left to right, moving down, and then back again from right to left until the entire paper was evaluated. We identified microplastics via visual inspection using criteria that has remained relatively constant across studies (Hidalgo-Ruz et al. 2012). To be classified as microplastic, particles must not have and visible cellular structures, must be equally thick along their length, and must have a clear consistent color. Of course, exceptions to these rules occur. We observed exceptions similar to those shown in the Marine Environmental Research Institute microplastics identification guide. Some plastics were frayed at the end, thus not being exactly the same thickness for the entire length. Also, multicolor plastics were identified that were not one constant color. We classified each plastic that we located by type (primary or secondary), shape (bead, nurdle, fiber, fragment, film, sheet, or foam), and color.

Due to the COVID-19 pandemic, we moved the quantification of microplastics on filter papers out of the laboratory for a short period. During this time, we took further precautions to minimize contamination. We conducted counts in a tent fitted with an air filter to remove any airborne particles. We allowed this filter to run for 30 minutes prior to any quantification. Once again, we wore a lab coat and nitrile gloves at all times. We also kept samples covered in their respective petri dishes at all times.

Statistical Analysis

From each set of three gizzard portions in a sample, we calculated the raw number of microplastics as well as concentration. We calculated this concentration by dividing the number of microplastics found on each sample by the mass of the gizzard portion. Across all samples, we calculated the frequency of occurrence as the percentage of birds that contained any microplastics in their gizzards. We also calculated the percent of each plastic shape, type, and

color. To determine significant differences in median microplastic count and concentration between sex, location, and feeding ecology, we ran a series of Mann-Whitney Rank Sum Tests because the data were not normally distributed. We performed Mann-Whitney Rank Sum Tests as opposed to T-tests due to the lack of normality in our data. Because of the low sample size of dabbling ducks (n=1), feeding ecology comparisons were only made between diving ducks and Canada Geese. In addition to these comparisons, we used a Fisher's Exact Test to compare the proportion of ducks to geese with microplastics in their gizzards. We did all tests and calculations in either R Studio v 1.2.1335 or Microsoft Excel v 16.47.1.

Results

Overall, we processed a total of 28 birds and found microplastics in 15 of their gizzards (frequency of occurrence= 53.6%). We extracted a total of 29 microplastics from them (Fig 2). All of these plastics were secondary microplastics. Most of them were fibers (82.8%), but some were fragments (17.2%) (Fig 3A). The most prevalent color we found was blue (41.4%), but other common colors included red (20.7%) and black (20.7%) (Fig. 3B). The plastic counts and concentrations between specimens were highly variable. Plastic counts ranged between 0-4 particles per bird, while concentration ranged from 0-1.75 plastics/gram of gizzard content. The highest concentration came from a male Ringneck Duck in Culpeper County.

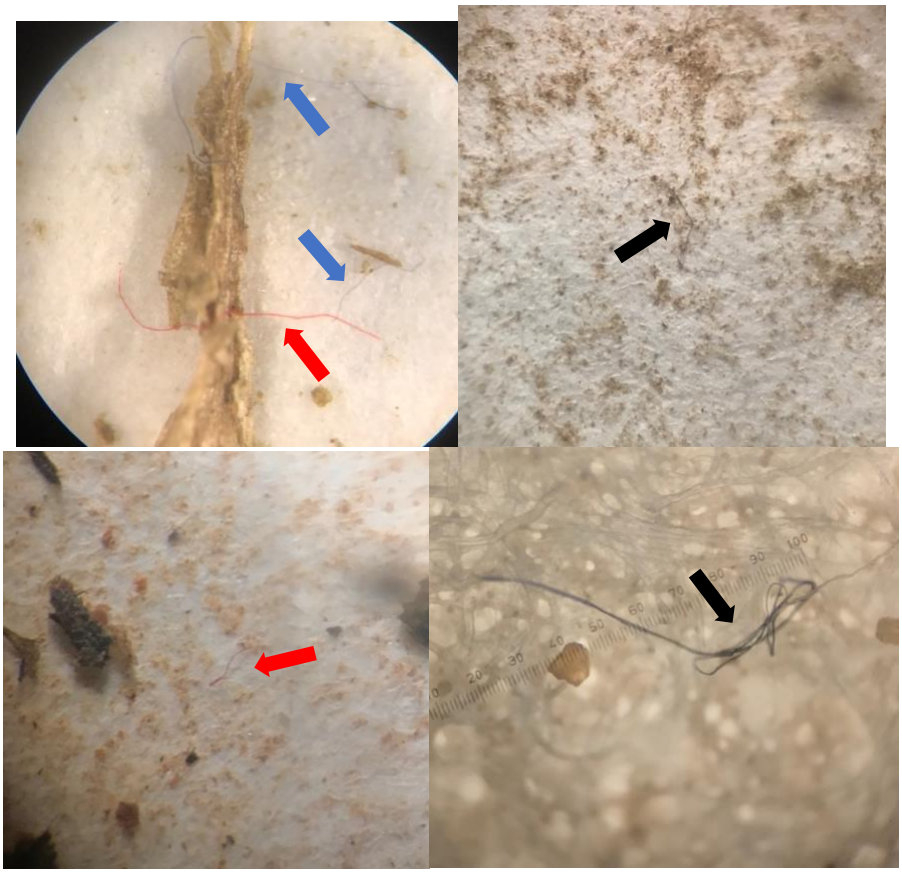

Figure 2: Photographs of microplastic fibers found in gizzard samples. Arrow colors correspond with the color of the microplastic photographed.

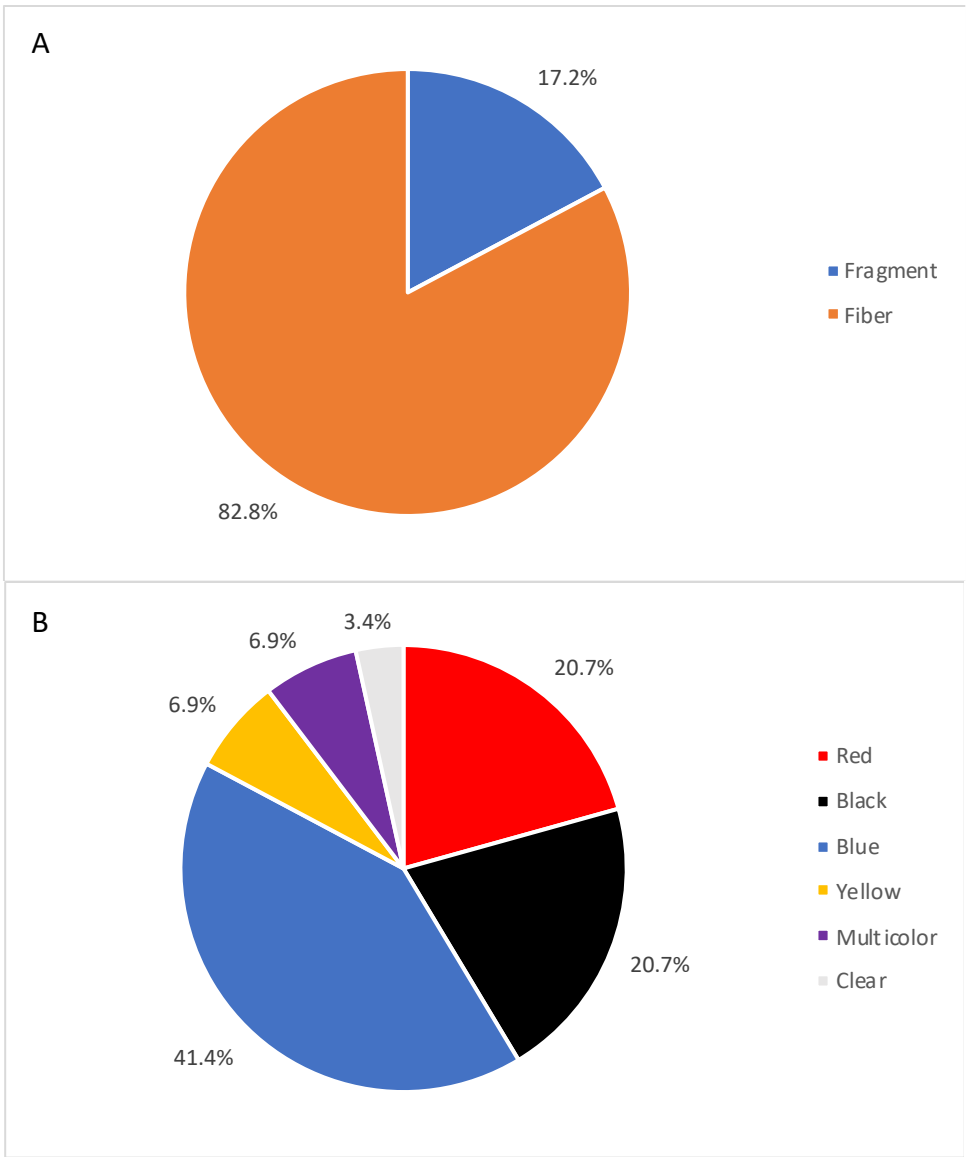

Figure 3: Percentages of each plastic shape (A) and color (B). 3A: the most abundant plastic shape was by far fibers and some fragments were found as well. 3B: The most abundant plastic color was blue, but other colors included black, red, yellow, multicolor, and clear.

Median microplastic count or concentration did not significantly differ between sexes (Raw count: Mann-Whitney Rank Sum Test W=27.5, p=0.65, Concentration: Mann-Whitney Rank Sum Test W=21, p=0.26) or locations (Raw count: Mann-Whitney Rank Sum Test W=102.5, p=0.84, Concentration: Mann-Whitney Rank Sum Test W=125.5, p=0.19) (Table 1). Upon comparing plastics between diving ducks and Canada Geese, microplastic count was not significantly different (Mann-Whitney Rank Sum Test W=108.5, p=0.22), but concentration was

(Mann-Whitney Rank Sum Test W=134, p=0.01). Diving ducks on average had a higher median microplastic concentration (0.395 plastics/gram) than Canada Geese (0 plastics/gram) (Fig. 4, Table 1). While we were unable to add dabbling ducks to this comparison, we were able to sample one Mallard from Culpeper County. This Mallard had a plastic count of one fiber and a concentration of 0.44 plastics/gram. Ducks and geese did not differ in their proportions of individuals with microplastics detected in their gizzards and those without (Fisher's Exact Test p=0.14). More geese appear to have ingested microplastics compared to ducks, but larger sample sizes are needed to determine significance (Fig. 5, Table 2)

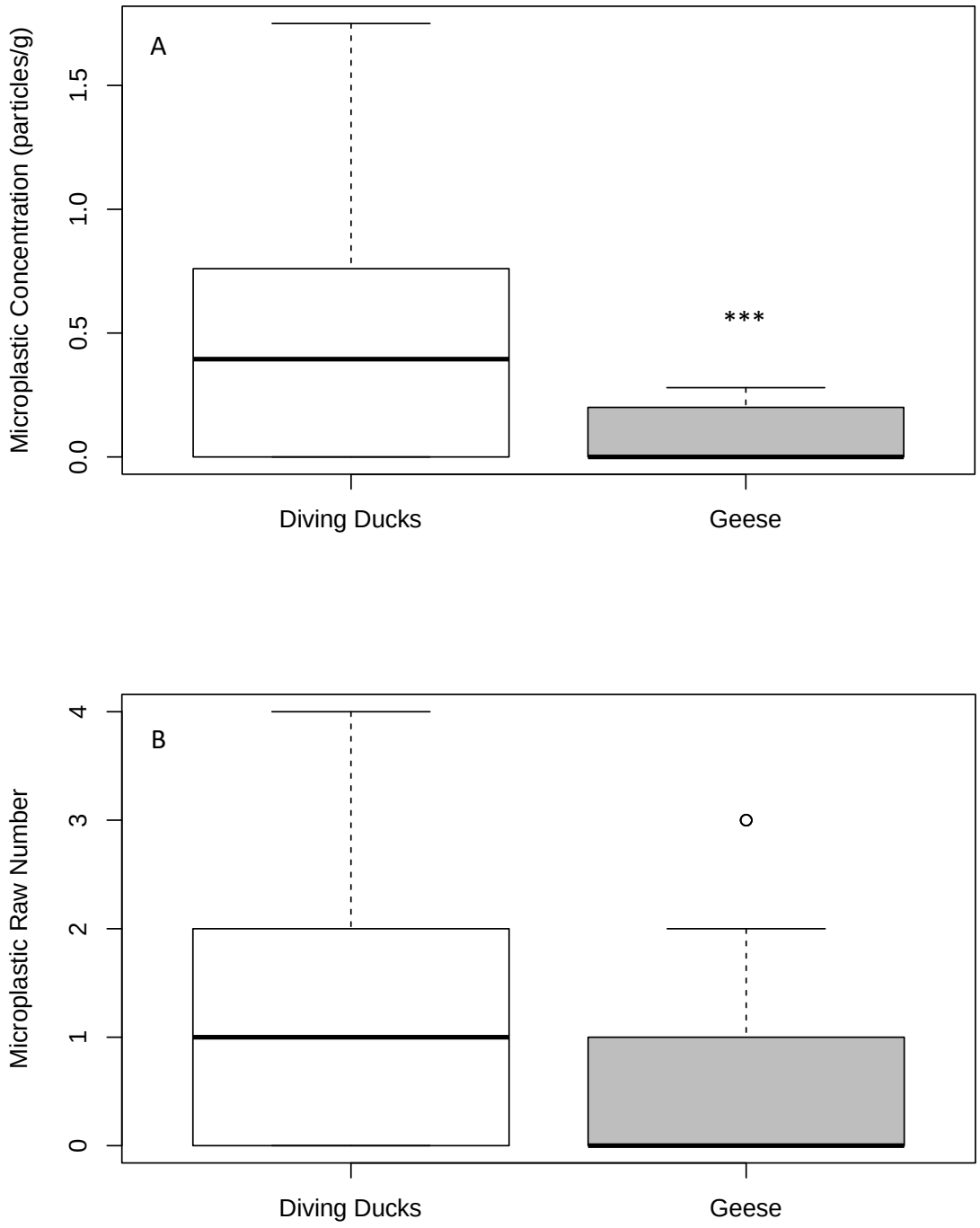

Figure 4: Comparisons of microplastic concentration (A) and count (B) between diving ducks (n=10) and Canada Geese (n=17). Microplastic concentrations of gizzard content samples significantly differed between diving ducks and geese (Mann-Whitney Rank Sum Test W=134, p=0.01) while raw microplastic counts did not (Mann-Whitney Rank Sum Test W=108.5, p=0.22).

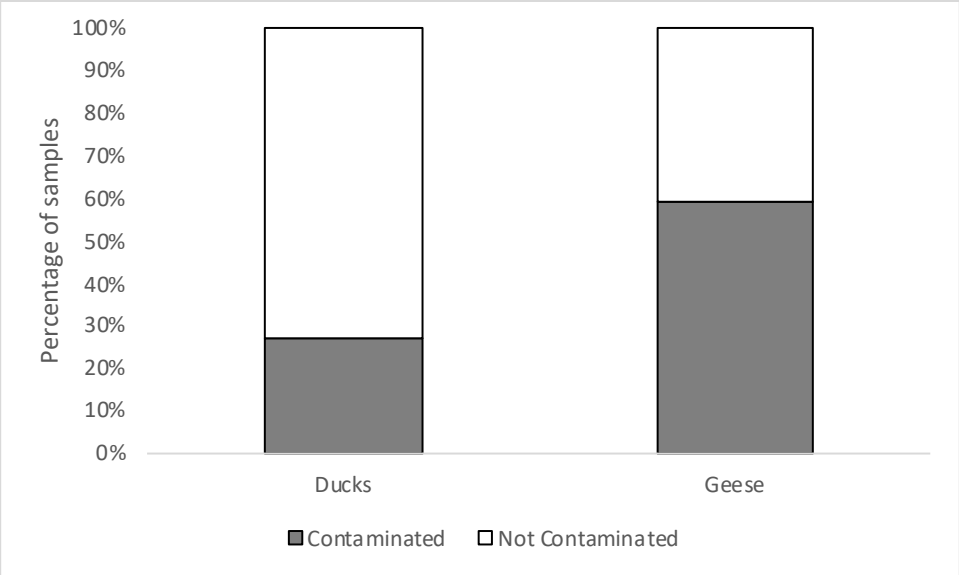

Figure 5: Comparison of the proportion of ducks (n=11) and geese (n=17) that have or have not consumed microplastics. Neither geese nor ducks had a significantly higher proportion of individuals with microplastics in their gizzards (Fisher's Exact Test p=0.14).

Table 1: Descriptive statistics of each Mann-Whitney Rank Sum Test comparison. We made comparisons between medians for the Mann-Whitney Rank Sum Test. Means and Standard deviations are shown as well.

Variable	Group	N	Median count	Median conc.	Mean count	Mean conc.	Count sd	Conc. Sd
Sex	Male	8	1	0.4	1.13	0.5	1.25	0.57
	Female	8	0.5	0.06	1	0.28	1.41	0.44
Location	Culpeper	14	1	0.21	0.93	0.36	1.07	0.48
	Westmoreland	14	0	0	1.14	0.18	1.51	0.32
Feeding ecology	Diving duck	10	1	0.4	1.4	0.55	1.51	0.57
	Canada goose	17	0	0	0.82	0.09	1.19	0.11

Table 2: Proportions of polluted to not polluted ducks (n=11) and geese (n=17) used in the Fisher's Exact Test.

Group	N	Proportion
Ducks	11	3/8
Geese	17	10/7

Discussion

Overall, our results provide evidence that microplastics are consumed and retained in the digestive tracts of some Virginia waterfowl. We determined here that neither raw microplastic

count nor microplastic concentration were significantly different between both sex and location. Additionally, while microplastic count did not differ between dabbling ducks and Canada Geese, microplastic concentration did. This indicates that these birds are not consuming different amounts of microplastics, but the diving ducks are ingesting microplastics at a higher rate. These results could be due to a few factors. Diving ducks are more omnivorous than Canada Geese, and will consume organisms such as bivalve mollusks. Bivalves and other filter feeders are efficient at ingesting microplastics and microparticles as they filter for food (Davidson and Dudas 2016, Su et al. 2018, Ward and Kach 2009) and may potentially concentrate particles with ease. Therefore, these diving ducks may be secondarily consuming high concentrations of plastics via their diet. To our knowledge, no studies assessing freshwater birds have measured plastic concentration prior to this one. These results highlight the importance or taking the amount of gizzard content in a sample into account during analysis, as microplastic ingestion may be partially a function of overall food consumption. We obtained one dabbling duck specimen: a Mallard. While its concentration was fairly low compared to other ducks (concentration=0.44 particles/gram), more dabbling ducks must be collected before we can draw any conclusions.

While no other study we found assessing freshwater birds measured microplastic concentration per unit mass of gastrointestinal tract contents, we can still compare our results to those found elsewhere. For example, our frequency of occurrence (15/28 or 53.6%) was fairly high when compared to other studies. One study done in Canada assessed 350 birds and found microplastics in 15 of them (4.3%) (Holland et al. 2016). However, this percentage included a species of sea duck, so the percentage of freshwater species was slightly lower than what was reported. Winkler et al. 2020 found that 10 out of 133 (7.5%) Kingfisher pellets in northern Italy contained microplastics. Not all frequencies were quite this low. A study investigating cormorant

chicks near the Great Lakes found that 26 out of 30 chicks had ingested microplastics (86.7%) (Brookson et al. 2019). Studies showing lower frequencies of occurrence assessed many more samples than those with higher frequencies, including our own. We need to assess more gizzards in order to get a clearer understanding of ingestion levels.

Raw numbers of microplastics detected in our study can also be compared with previous studies. We found a total of 29 microplastics in our 28 gizzards. This result can be compared with a study done in South Africa that found a total of 82 microplastic fibers in 691 samples (Reynolds and Ryan 2018). If we proportionally scale our results to theirs, we would have found about 715 fibers in 691 samples. On the higher end of these results is a study assessing fecal material in the Spanish lakes that found 736 plastics in 228 samples (Gil-Delgado et al. 2017). This number is extremely high compared to ours. If we scale our results again, we would have found about 236 plastics in 228 samples. The reasons for the differences observed between these studies could be numerous. The differences could be due to the species assessed. These studies cover a multitude of birds from herbivores to piscivores. Each species has its own feeding ecology, which could impact the amount of microplastics they consume as has been speculated with other organisms (Wright et al. 2013). The level of urbanization may also partially explain differences in results. The counties in our study have a fairly high level of urbanization compared to some of the studies conducted in areas with lower microplastic prevalence. Holland et al. (2016) collected their waterfowl specimens from some fairly isolated areas in Canada, which may explain their low frequency of occurrence and microplastic count. Conversely, Gil-Delgado et al. (2017) collected their waterfowl specimens from areas that used to be garbage dumping grounds, which may explain their high microplastic count. The differences observed may be due to differences in methods used across each study. A general issue across microplastics studies is

the lack of standardization of methods. Differences in equipment used, part of the organism analyzed, and units of measurement reported in results make it difficult to compare studies and understand consequences of microplastic consumption (Provencher et al. 2017). Not every freshwater bird study examined gizzard contents as we did. Others examined fecal material, feather brushings, and even the entire gastrointestinal tract. Additionally, not every study used chemical digestion and density separation, instead opting for the usage of sieves or the disaggregation of samples with water to separate microplastics from organic debris (Gil-Delgado et al. 2017; Holland et al. 2016; Reynolds and Ryan 2018). Even so, studies that used chemical digestion did not use the exact reagents we used. While we used hydrogen peroxide, Brookson et al. (2019) used potassium hydroxide. All of these differences in methods could impact results and their interpretation. Future work should take care to standardize methods as much as possible.

These plastics were exclusively secondary and almost exclusively fibrous, which is what other studies on freshwater bird species have determined as well. Reynolds and Ryan (2018) found that 100% of their plastics were fibers, and Winkler et al. (2019) found that all but one plastic particle was a fibrous shape. The other microplastic was a fragment. Microplastic fibers tend to be one of the most commonly identified microplastic shapes in the environment, which may be making them more available to the freshwater birds in these studies (Horton et al. 2016). Because of their shape and ability to remain and accumulate in the intestinal tract for long periods, microplastic fibers may be more dangerous to organisms than other plastic shapes, potentially making microplastic ingestion more impactful for freshwater birds given the prevalence of fibers in their systems (Ma et al. 2019). Determining the sources of these microplastics is difficult, as they are numerous and widespread. Since all of these microplastics

were secondary, they resulted from the breakdown of larger plastic fragments, but pinpointing what these larger fragments were is difficult. Fibrous microplastics typically originate from the breakdown of synthetic clothing and other fabrics, whereas fragments come from a variety of sources, including bottles, plastic furniture, broken down tires, and many more (Rochman et al. 2019). Future studies should attempt to classify plastics and pinpoint origins as to determine which sources need further regulation. However, determining exact sources is more difficult for fibers and fragments as opposed to other plastic shapes (Helm 2017).

There are some commonly cited mechanisms behind how microplastics enter the environment initially. The most common are wastewater treatment plants, as they are capable of releasing numerous microplastics into the environment via their effluent (Mason et al. 2016). However, the level of microplastic release is highly dependent on the efficiency of the wastewater treatment plant itself. Some studies have found that if the plant is efficient enough, then microplastic release is minimal (Carr et al. 2016). Another commonly stated source, especially for fibrous microplastics, is atmospheric deposition. Microplastics can enter the atmosphere and be deposited over great distances. One study determined that a daily average of 365 microplastics per meter squared could deposit on a remote mountain catchment in France and that some of these plastics had traveled over 95km (Allen et al. 2019). Another likely source is runoff of terrestrial microplastics into freshwater systems (Horton et al. 2016). Interestingly, another potential source is from birds' gizzards themselves. Some soil organisms (which possess organs that are analogous to bird gizzards) may be capable of breaking down and creating more microplastics in their environment (He et al. 2018). Given that the purpose of a gizzard is to break down food items a bird swallows, it may also aid in the production of secondary microplastics in not only the bird, but the environment as microplastics are excreted. This means

that beyond the possibility of microplastics harming the waterfowl assessed here, the waterfowl themselves may be further degrading larger plastic fragments and releasing them as microplastics.

As has been shown in other studies assessing differences in macroplastic loads between sexes (Spear et al. 1995), we found no significant differences in microplastic number or concentration between males or females. This result may be due to our small sample size. We may expect major differences between male and female waterfowl during breeding and molting periods, which seems to be when differential feeding rates between sexes occur. A study assessing dabbling ducks found that generally, female dabbling ducks feed more than their mates during the breeding season, which may have to do with their greater energetic demands of reproduction (Kaminski and Prince 1981). Additionally, a study assessing monogamous Cackling Canada Geese found that females forage more prior to molting than males do (Sedinger and Raveling 1990). Our birds were collected outside of their breeding and molting seasons, so we may need to resample birds during these sensitive periods to see a difference.

Furthermore, we found no significant differences in microplastic count or concentration between Culpeper or Westmoreland County. We hypothesized that Westmoreland County would have a higher microplastic concentrations since it is downstream from high population areas along the Potomac. While that may have contributed to our fairly high results, it was not higher than values in Culpeper County. Culpeper County has a higher human population (52,605 in 2019) that Westmoreland (18,015 in 2019), and additionally, has more smaller reservoirs situated near urbanized areas (US Census Bureau, 2019). These lakes may concentrate microplastics in a similar way to what was observed in a remote mountain lake depending on water residence times (Free et al. 2014). Another possibility is that the ducks and geese collected in Westmoreland

were not collected near the Potomac. When our specimens were donated, no specifics as to where in Westmoreland or Culpeper the bird originated from were provided. Further studies should take care to specify location of specimen collection in greater detail.

The toxicological impacts of microplastics in these specific quantities on these waterfowl is mostly unknown. Most of the studies that have investigated microplastics' effects on organisms have been done in a laboratory setting on small invertebrates. While we cannot be certain of the exact impacts of these plastics on waterfowl, we can make some predictions. The most probable impact that these microplastics have on waterfowl is tissue contamination by absorbed pollutants and plasticizers. A study on short-tailed shearwater carcasses showed that pollutants transported by plastics are bound to tissues (Tanaka et al. 2013), meaning that similar impacts are likely suffered by waterfowl. We collected tissue samples from the waterfowl during dissection, meaning we can assess them for contaminants in a future study. Smaller species of waterfowl may be more susceptible to microplastic contamination than larger species. We demonstrate here that when gizzard sample volume is taken into account, diving ducks have higher microplastic concentrations than Canada Geese despite the fact that they consumed about the same number of particles. Because ducks have smaller gizzard sample masses (diving ducks: 0.86±0.3g, Canada Geese: 3.23±1.15g), a given plastic takes up more space and may cause more harm. It is also possible that these plastics are not causing much harm and only pass through the digestive tract. None of the plastics in this study could be observed without a microscope, meaning they were likely much too small to cause much physical damage. Additionally, the microplastics quantified here were quite scarce and perhaps were causing minimal if any damage. However, no studies to our knowledge have investigated the impacts of microplastic ingestion on waterfowl of any kind. Future work should attempt to show how plastics in these

quantities and with these characteristics impact birds, which would allow more accurate determination of the threats microplastics pose them.

This study had some limitations. The salt used during density separation (NaCl) is not as dense as other types of salt used in microplastic analysis such as NaI or $ZnBr_2$ (Quinn et al. 2017). Therefore, we were likely unable to extract plastics with more dense polymers. While the use of NaCl limits our estimates of pollution, we note that these dense salts are not only expensive, but also toxic (Quinn et al. 2017). We therefore selected cheaper and more environmentally benign salt at the cost of potential loss of dense plastic polymers. We did not extensively use any chemical analysis to quantify these plastics. While we preliminarily used both infrared spectroscopy and fluorescence, the results reported here are all from visual inspection. Visual inspection is often not as accurate as other microplastic quantification methods, as non-plastic particles are often mistaken for plastic (Eerkes-Medrano et al. 2015). While we were careful and conservative during our visual identifications, our lack of chemical analysis should be noted. Additionally, the sample size of this study is still quite low, especially for dabbling ducks. Increasing our sample size of dabbling ducks would allow for their inclusion in the feeding ecology comparisons. Furthermore, increasing sample sizes overall would allow for us to determine potential statistical interactions between our independent variables.

Overall, the results of this study show that waterfowl from Virginia not only consume microplastics, but retain them in their digestive tracts. In the future, we want to assess these plastics chemically in order to more accurately determine microplastic prevalence. We also want to collect more diving ducks so they may be included in this analysis. We predicted that they would have higher concentrations than both diving ducks and geese due to their feeding ecology, but we must collect more samples to support or reject this. Additionally, we want to assess the

difference in concentration between resident and migratory geese. The Canada Goose is a partial migrant, meaning populations of non-migratory, or resident geese currently inhabit much of the United States. These populations are a result of efforts to reestablish populations back in the 1960s (Mowbray et al., 2002). Migratory geese breed in remote northern North America while resident geese stay near fairly urbanized locations year-round. Despite this, no studies have investigated if microplastics are present in different concentrations between migratory and non-migratory geese. We did manage to collect both resident and migratory geese in this study, but they were confined to separate locations. All of our resident geese came from Culpeper County while our migratory geese came from Westmoreland County, so a comparison between the two groups would have been confounded by location. Any future study comparing resident and migrant geese should be careful to control for location. As microplastic concentrations inevitably increase, these contaminants will pose a greater threat to organisms and ecosystems. Even if all plastic production were to halt today, the degradation of current pollution will cause microplastic concentrations to rise for years to come (Barnes et al. 2009). We must continue to monitor prevalence in order to keep track of this relatively novel issue.

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